APPENDIX B

ANTISENSE AND NUCLEIC ACID DRUG DEVELOPMENT 12:411–418 (2002)

Brief Communication

The Effectiveness of Double-Stranded Short Inhibitory RNAs (siRNAs) May Depend on the Method of Transfection

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ABSTRACT

RNA interference (RNAi) is a recently described powerful experimental tool that can cause sequence-specific gene silencing, thereby facilitating functional analysis of gene function. Consequently, we became interested in using RNAi to determine the function of aberrantly expressed ErbB3 in the KAS-6/1 human myeloma cell line. Despite the wealth of information available on the use of RNAi, dsRNA target design, and the transfection of dsRNA *in vitro*, little information is available for transfecting dsRNA into nonadherent cells from any species. In the present study, we report that gene silencing of ErbB3 was not observed in myeloma cells when dsRNA targeting ErbB3 was introduced using conventional transfection agents and protocols that have proved successful for several adherent cell lines. Silencing of ErbB3, however, was observed in T47D cells, an adherent breast carcinoma cell line, using the same transfection methods, indicating that our target sequence was functional for gene silencing of ErbB3. Interestingly, ErbB3 was silenced in myeloma cells when the dsRNA target was introduced by electroporation. Thus, our studies illustrate the striking dependence of dsRNA-mediated gene silencing in some cells on the methods of dsRNA transfection.

INTRODUCTION

RNA INTERFERENCE (RNAi), a phenomenon first discovered in *Caenorrhabditis elegans*, is a response to double-stranded RNA (dsRNA) that can cause sequence-specific gene silencing (Fire et al., 1998). At present, the mechanism of this phenomenon is not entirely understood, but current models propose the involvement of two distinct steps (Hutvagner and Zamore, 2002). The first, initiation step involves cellular uptake or input of long strands of dsRNA and the subsequent enzymatic digest of these strands into 21–23-nucleotide (nt) small interfering RNAs (siRNAs), which are often referred to as "guide RNAs" (reviewed in Hannon, 2002). In the second, effector step, the guide RNAs bind to the RNA-induced silencing complex (RISC), a nuclease complex proposed to have helicase activity, endonuclease activity, and homology searching activity (Hammond et al., 2001). Consequently, it is believed that the RISC complex is able to unwind the bound double-stranded guide RNA and then targets the homologous endogenous transcript via base pairing interactions. The endogenous transcript is subsequently cleaved by the RISC complex, resulting in downregulation or silencing of the gene.

The utility of this discovery for quickly and easily determining the function of a gene was immediately recognized and led to an examination of the ability of chemically synthesized siRNA duplexes to silence genes of interest. Indeed, several groups have now described successful results of specific gene silencing using siRNA

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duplexes (Dudley et al., 2002; Harborth et al., 2002; Li et al., 2002; Matuliene and Kuriyama, 2002; Tijsterman et al., 2002; Tuschl et al., 1999). Because of its early success, RNAi has become one of the hottest techniques since the invention of PCR. Consequently, we became interested in using RNAi to determine the function of aberrantly expressed ErbB3 in the KAS-6/1 human myeloma cell line. It is noteworthy, however, that successful use of RNAi in human cells, with only one exception (Wilda et al., 2002), has been accomplished using adherent cells. Our goal in this study was to further explore the feasibility of using RNAi in nonadherent human cells. In this report, we describe the methods that allowed us to use RNAi to silence ErbB3 in this cell line. Our results clearly illustrate that gene silencing using RNAi in this nonadherent cell line is critically dependent on the methods used to deliver RNA duplexes into the cell.

MATERIALS AND METHODS

Cell lines, culture medium, and reagents

The myeloma cell line, KAS-6/1, was derived from primary patient myeloma cells and has been described previously (Westendorf et al., 1996). This cell line was maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin G, 50 μg/ml gentamicin, 100 μg/ml streptomycin, 2 mmol/L glutamine, and 1 ng/ml interleukin-6 (IL-6) (Novartis Pharma AG, Basel, Switzerland). The breast adenocarcinoma cell line T47D was purchased from ATCC and maintained in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin G, 50 μg/ml gentamicin, 100 μg/ml streptomycin, and 2 mmol/L glutamine. Oligofectamine, Cellfectin, and DMRIE-C were purchased from Invitrogen (Carlsbad, CA).

Flow cytometry

To assess successful transfection of the fluorescein-labeled luciferase-specific siRNA duplex, cells were washed three times in ice-cold phosphate-buffered saline (PBS)/2% FBS to remove any surface-bound complex and then fixed in 1% paraformaldehyde. Immunofluorescence was detected using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). For measuring ErbB3 expression, 1×10^6 cells were incubated with an ErbB3 monoclonal antibody (mAb) (Neomarkers, Inc., Fremont, CA) for 30 minutes on ice in PBS/2% FBS, washed, and then incubated with phycoerythrin (PE)-conjugated goat antimouse IgG (Biosource International, Camarillo, CA) for another 30 minutes. Expression of gp130, insulin-like growth factor-I (IGF-IR), and VLA-4 (α4 integrin) was assessed via incubation with either PE-conjugated or FITC-conjugated receptor-specific Abs (Immunotech, Marseille, France; Molecular Probes, Eugene, OR; Serotec, Oxford, U.K., respectively) for 30 minutes on ice. Syndecan-1 expression was determined using an FITC-conjugated CD138 mAb (Serotec). All cells were then washed, fixed in 1% paraformaldehyde, and analyzed for immunofluorescence on a FACScan flow cytometer. To distinguish between intracellular and extracellular fluorescence, cells were incubated with or without a 0.2% solution of trypan blue for 5 minutes (Van Amersfoort and Van Strijp, 1994). Cells were washed twice with PBS and fixed as usual. The collected data were analyzed using WinMDI 2.5 software. The change in mean fluorescence intensity (ΔMFI) was calculated by dividing the MFI resulting from staining with the receptor-specific Ab by the MFI resulting from staining with the isotype control Ab.

Immunoprecipitation and immunoblotting

Following transfection, myeloma cells were cultured in RPMI supplemented with 10% FBS and IL-6, and breast carcinoma cells were cultured in DMEM medium supplemented with 10% bovine serum albumin (BSA) for 24, 48, 72, or 96 hours. Cells (5×10^6) were lysed in lysis buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1 mM EDTA, 15 mM sodium molybdate, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 2 mM Na_3VO_4, and 1 mM PMSF. Lysates were cleared of insoluble material by centrifugation for 10 minutes at 14,000 rpm. Whole cell lysate (75 μg) was diluted 1:1 with 2× SDS loading buffer, heated to 100°C for 5 minutes, resolved by SDS-PAGE, and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) for immunoblotting. Membranes were blocked for 1 hour in 25 mM Tris-HCl, pH 7.2, 150 mM NaCl, and 0.2% Tween (TBST) supplemented with 2% BSA. Immunoblotting for ErbB3 was detected by a 1:1000 dilution of anti-ErbB3 mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and horseradish peroxidase (HRP)-conjugated polyclonal antirabbit IgG (Amersham Pharmacia Biotech Inc., Piscataway, NJ) was used as the secondary at a dilution of 1:3000. Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) detection system (Super Signal, Pierce Biotechnology, Inc., Rockford, IL) and autoradiography.

Source of dsRNA molecules and transfection using Oligofectamine, Cellfectin, and DMRIE-C

The pGL2 siRNA duplex (target 5′-CGTACGCGGAATACTTCGA-3′), ErB3 siRNA duplex (target 5′-AACCAATACCAGACACTGTAC-3′), IGF-IR siRNA duplex (target 5′-CTACGCCCTGGTCATCTTC-3′), and survivin siRNA (target 5′-ACTGGACAGAGAAA-

GAGCC-3′) were synthesized and purchased from Dharmacon Research, Inc. (Lafayette, CO). The synthesis method was carried out according to the method described by Elbashir et al. (2001), in which a 21-nt sense and a 21-nt antisense strand are paired in a complementary manner. This pairing results in a target-specific 19-nt duplex region and a 2-nt overhang (dTdT for all the duplex RNAs used in this study) at the 3′-end. Duplexes labeled with fluorescein moieties were modified at the 5′-end of the sense strand. Transfection of these duplexes using Oligofectamine, Cellfectin, or DMRIE-C was carried out according to the procedure provided by Dharmacon (*www.dharmacon.com/siRNA.html*), which is also the procedure used by Elbashir et al. (2001). RNAi was assayed by flow cytometry after 24, 48, and 72 hours.

Transfection via electroporation

On the day before transfection, cells were split and cultured in RPMI supplemented with 10% FBS and IL-6. On the day of transfection, cells were spun down and washed once in Opti-MEM (Life Technologies Inc., Rockville, MD) before resuspension at a concentration of 20×10^6 cells or 400 μl. Cells were then incubated with 20 μg ErbB3-specific siRNA or the control fluorescein-labeled luciferase siRNA duplex for 10 minutes at ambient temperature in a 0.4-cm electroporation cuvette (BTX Genetronics, San Diego, CA). The cell/siRNA duplex mixture was pulsed once for 20 milliseconds at 250 V using a square wave electroporator (BTX Genetronics) and incubated at ambient temperature for 20 minutes prior to transferring cells to 6-well plates (Becton Dickinson) containing RPMI + 20% FBS + IL-6. RNAi was assayed by flow cytometry or Western blot analysis or both after 24, 48, 72, or 96 hours.

RESULTS AND DISCUSSION

We have had a longstanding interest in understanding the mechanisms underlying growth control of multiple myeloma cells. In this regard, we have employed a panel of disease-representative human myeloma cell lines that collectively display heterogeneity in cytokine responsiveness. Recent work in our laboratory focusing on receptor transactivation in this tumor model (French et al., 2002) led to the serendipitous discovery that ErbB3, a member of the epidermal growth factor receptor (EGFR) family, is atypically expressed in the multiple myeloma cell line, KAS-6/1 (D.K. Walters et al., unpublished observations). This finding was intriguing to us, as the KAS-6/1 cell line has also been shown to display an atypical growth response to interferon-α (IFN-α) (Westendorf et al., 1996). Consequently, we were interested in exploring the resulting phenotype of the KAS-6/1 cell line in the absence of ErbB3. Thus, the goal of the current study was to determine if RNAi could be used to induce gene silencing of ErbB3 in the myeloma cell line KAS-6/1.

As a first step, we designed an siRNA duplex that would specifically target ErbB3 using previously published guidelines for target design (Tuschl et al., 1999; Elbashir et al., 2001). Although siRNA duplexes have been shown to be effective at inducing RNAi in a number of adherent cell lines (Tuschl et al., 1999; Dudley et al., 2002; Tijsterman et al., 2002), successful use of this technology in nonadherent cells, for example, myeloma cells, had not been reported when we first began these studies. Therefore, before transfection of ErbB3 siRNA, it was first necessary to determine if the nonadherent KAS-6/1 cells could be transfected with dsRNA. To begin to address this question, we used several transfection protocols and tested our ability to transduce the KAS-6/1 cells with a preselected fluorescein-labeled duplex that targets the firefly luciferase plasmid (pGL2-Control) used by Elbashir et al. (2001). We chose this duplex because of our ability to assess transfection efficiencies by flow cytometry and because the KAS-6/1 cells do not express firefly luciferase and, therefore, should be unaffected by this dsRNA. We first employed Oligofectamine as a transfection reagent. We also used the same procedure described by Elbashir et al. (2001) that allowed these investigators to successfully transfect luciferase siRNA duplexes into a variety of luciferase-expressing adherent cell lines, including NIH/3T3, COS-7, HeLa S3, and 293 cells. In addition, we also evaluated the transfection ability of Cellfectin and DMRIE-C, a transfection reagent that is advertised as being specific for suspension cells. Fluorescence intensity was assessed by flow cytometry 24 hours after transfection. As seen in Figure 1A, an increase in fluorescence was detected in the KAS-6/1 cell line using all three transfection agents (Cellfectin, 59%; Oligofectamine, 66%; DMRIE-C, 77%). Of these three agents, however, DMRIE-C was clearly the most effective. As expected, no increase in fluorescence was observed when cells were incubated with siRNA in the absence of transfection agent. We concluded from these results that the fluorescein-labeled siRNA luciferase duplex could be successfully transfected into the KAS-6/1 cell line and that this transfection was most successful using DMRIE-C. Thus, DMRIE-C was used in subsequent experiments involving a transfection agent. Using all three reagents, we also assessed the effects of altering cell number or transfection agent concentration, and neither of these adjustments had a significant effect on transfection efficiency (data not shown).

The results demonstrated our ability to transduce labeled dsRNA into KAS-6/1 cells. We next wished to assess the ability of the ErbB3 siRNA duplex to silence

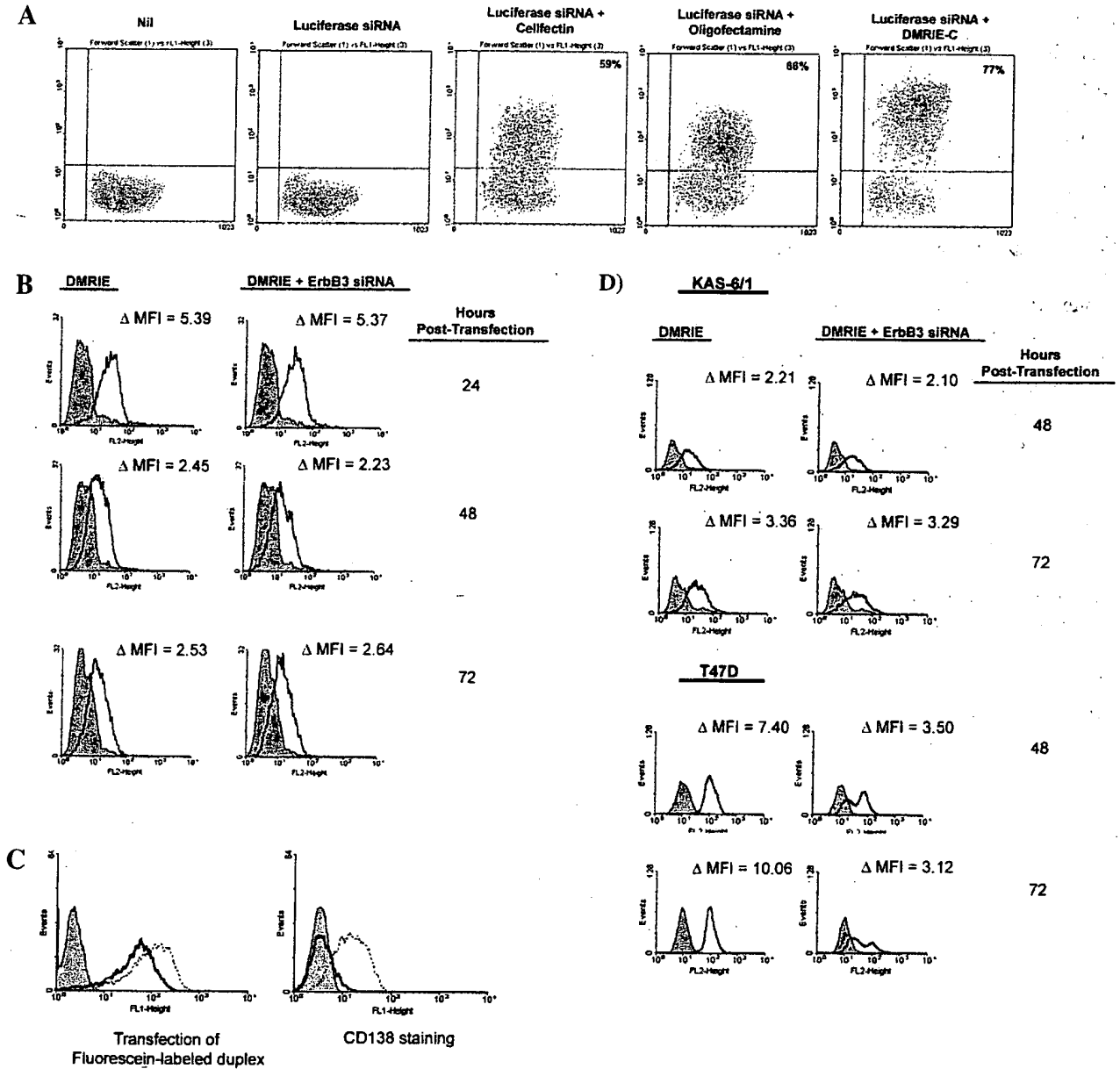

FIG. 1. Analysis of siRNA transfection efficiency and ErbB3 expression in myeloma cells following transfection with ErbB3 siRNA. (**A**) The KAS-6/1 myeloma cell line was transfected with fluorescein-labeled siRNA that targets the firefly luciferase plasmid. Fluorescence intensity was assessed by flow cytometry 24 hours after transfection. Nonspecific binding of siRNA to cells was assessed by incubating KAS-6/1 cells with siRNA in the absence of any transfection reagent (second panel from left). Forward scatter is shown on the X axis, and fluorescence intensity is shown on the Y axis. (**B**) KAS-6/1 cells were transfected with siRNA specifically targeting ErbB3 using DMRIE-C. Downregulation of ErbB3 surface expression was assessed 24, 48, and 72 hours after transfection using an ErbB3-specific mAb and flow cytometry. Isotype control, solid gray histogram; specific staining, solid line. (**C**) KAS-6/1 cells were transfected with fluorescein-labeled siRNA that targets the firefly luciferase plasmid. At 24 hours after transfection, cells were incubated with or without trypan blue before flow cytometric analysis. As a control, KAS-6/1 cells were incubated with an FITC-conjugated CD138 mAb and incubated with or without trypan blue prior to fixation. Isotype control, solid gray histogram; specific staining without trypan blue, dashed line; specific staining with trypan blue, solid line. (**D**) The KAS-6/1 myeloma cell line and the adherent T47D breast carcinoma cell line were transfected with siRNA specifically targeting ErbB3 using DMRIE-C. Downregulation of ErbB3 surface expression as assessed 48 and 72 hours after transfection via flow cytometry. Isotype control, solid gray histogram; specific staining, solid line.

ErbB3 expression, and we transfected the KAS-6/1 cells with the ErbB3 siRNA duplex using the protocol described and the DMRIE-C reagent. As a control, we also transfected cells with the fluorescein-labeled luciferase duplex. Overall transfection efficiency was assessed by FACS analysis of the percent positive fluorescent cells following transduction of the labeled duplex. Silencing of ErbB3 was assessed via flow cytometry. Similar to the previous experiments, an increase in fluorescence was detected following transfection of the fluorescein-labeled duplex (data not shown). However, when cells were transfected with the ErbB3-specific dsRNA complex, no significant decrease in surface expression of ErbB3 relative to mock-transfected cells could be detected (Fig. 1B). Thus, despite our ability to successfully transduce the labeled duplex, the ErbB3 siRNA duplex failed to alter ErbB3 expression. In additional experiments (results not shown), the ErbB3 siRNA duplex remained ineffective even after alteration of the duplex concentration, cell number, or transfection agent concentration.

Although the results shown in Figure 1A suggest that DMRIE-C was able to deliver dsRNA into the KAS-6/1

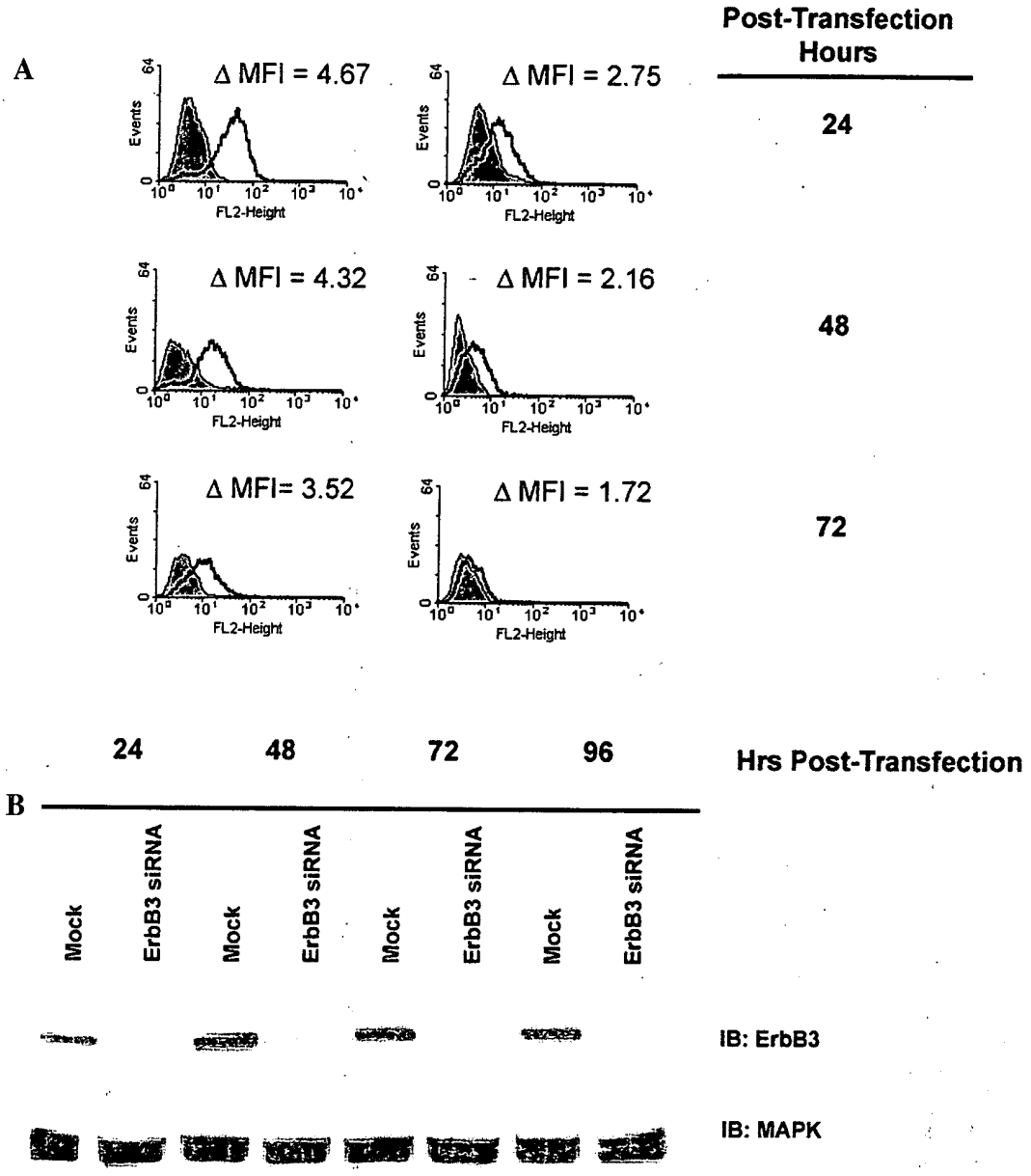

FIG. 2. Electroporation of KAS-6/1 cells with ErbB3-specific siRNA results in downregulation of ErbB3. (**A**) KAS-6/1 cells were transfected with ErbB3-specific siRNA via electroporation. Silencing of ErbB3 was assessed 24, 48, and 72 hours after transfection using an ErbB3 mAb and flow cytometry. Isotype control, solid gray histogram; specific staining, solid line. (**B**) Silencing of ErbB3 was also assessed by Western blot, following electrophoresis of whole cell lysates, using an ErbB3-specific Ab 24, 48, 72, and 96 hours after transfection.

cells, because the ErbB3-specific dsRNA complex was unable to modulate ErbB3 expression, it was possible that DMRIE-C simply facilitated cell surface binding of dsRNA but did not cause intracellular uptake. To address this possibility we again transfected the KAS-6/1 cells with the FITC-labeled duplex using DMRIE-C. Prior to fixation and flow cytometric analysis, we incubated the cells with or without trypan blue to assess extracellular vs. intracellular fluorescence. Of interest, although trypan blue was able to completely quench cell surface fluorescence as detected using an antibody to cell surface Syndecan-1 (Fig. 1C, right), trypan blue did not have a significant effect on the fluorescence signal emitted by KAS-6/1 cells transfected with the fluorescent dsRNA duplex (Fig. 1C, left). We conclude the majority of the duplex is successfully transfected into the KAS-6/1 cells using DMRIE-C and does not simply attach to the cell surface.

Although the manufacturer indicates that >70% of targets selected using the Tuschl et al. (1999) guidelines are successful in inducing RNAi of the respective gene of interest, it was possible that the ErbB3 siRNA duplex used in the studies described was simply an inefficient or ineffective ErbB3 target. To test this possibility, we trans-

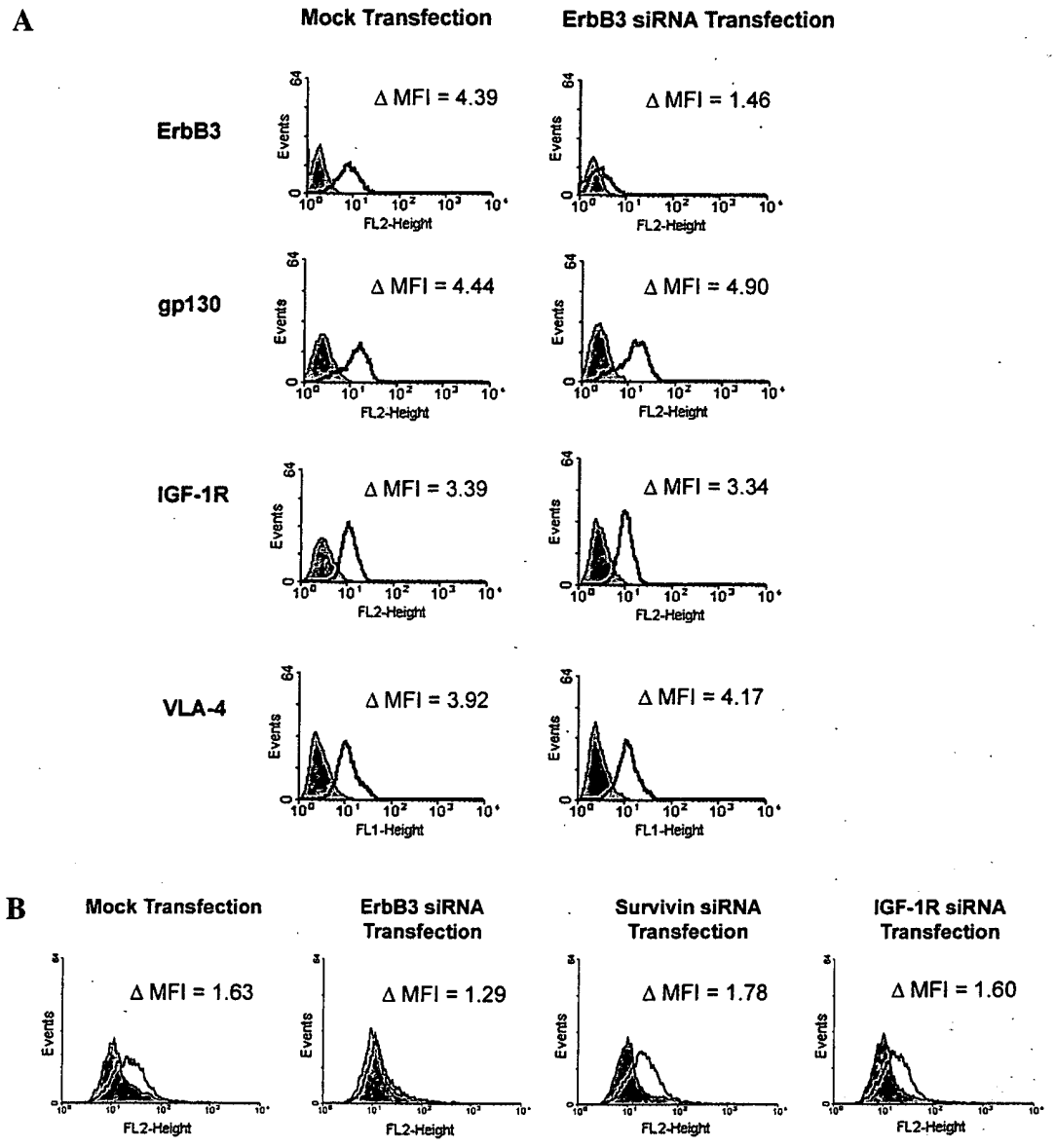

FIG. 3. Silencing of ErbB3 via RNAi is specific. (**A**) KAS-6/1 cells transfected with ErbB3 siRNA were assessed for expression of ErbB3, gp130, IGF-IR, and VLA-4 using receptor-specific Abs and flow cytometry 24 hours after transfection. (**B**) KAS-6/1 cells transfected with ErbB3 siRNA, survivin siRNA, or IGF-1R siRNA were assessed for downregulation of ErbB3 using an ErbB3-specific Ab and flow cytometry 24 hours after transfection.

fected T47D cells with the ErbB3 siRNA duplex using the transfection agent, DMRIE-C. T47D is an adherent breast carcinoma cell line that is known to express ErbB3. Gene silencing of ErbB3 was again assayed via flow cytometry using an ErbB3-specific antibody 48 and 72 hours after transfection. As shown in Figure 1C, transfection of the KAS-6/1 cell line with the ErbB3-specific siRNA duplex was again unsuccessful at silencing ErbB3 expression. However, significant downregulation of ErbB3 was observed in the T47D cells 48 and 72 hours after transfection. Thus, these data suggest that the ErbB3 siRNA target sequence is indeed functional at silencing ErbB3 and that its failure to silence ErbB3 in the KAS-6/1 cells must result from another factor(s).

To better understand why ErbB3-specific dsRNA duplex was ineffective in the KAS-6/1 cells, we again used the fluorescent duplex and used fluorescence microscopy to study intracellular localization after transfection. Of interest, we observed a cytoplasmic punctate staining pattern (results not shown). This pattern of localization to discrete foci is similar to the findings reported by Byrom et al. (2002) when they transfected fluorescently labeled c-*myc* siRNA into HeLa S3 cells. Similarly, when Coonrod et al. (1997) studied the efficiency of various DNA plasmid transfection methods, they reported that lipofection or Ca_2PO_4 transfection or both resulted in an initial granularlike accumulation of plasmid near the cell surface, with nuclear accumulation occurring over time. In contrast, it was reported that transfection via electroporation resulted initially in a more uniform staining of the cytoplasm. From these observations, Coonrod et al. concluded that the electroporation-induced pores most likely allowed more direct access to the soluble portion of the cytosol, whereas DNA transfected via lipofection may have to be sorted through endosomes and lysosomes before entering the cytoplasm or nucleus. It is possible, therefore, that although liposome-based transfection agents did permit siRNA duplex entry into the KAS-6/1 cell line, the accessibility of these duplexes to the RISC in these cells may be restricted.

To test this possibility, we next wished to determine if we could introduce the ErbB3 siRNA duplex into the KAS-6/1 cell line by electroporation. We have previously used electroporation to transduce expression plasmids into the KAS-6/1 cell line (French et al., 2002). We incubated the KAS-6/1 cell line with or without the ErbB3 siRNA duplex for 10 minutes and then electroporated the cells using 250 V for 20 milliseconds. There was striking downregulation of ErbB3, as revealed by flow cytometry and Western blot analysis, in the KAS-6/1 cell line as early as 24 hours after transfection (Fig. 2). Furthermore, this downregulation was maintained in the KAS-6/1 cell line over the 96-hour period. Alteration of voltage or siRNA duplex concentration did not have any significant effect on transfection efficiency or degree of silencing (data not shown). From these results, we concluded that the ErbB3 siRNA target is functional for silencing and that the RNAi pathway cannot be activated in the KAS-6/1 myeloma cell line with siRNA duplexes using cationic lipid or liposome-based transfection agents. However, transfection of siRNA duplexes targeting ErbB3 by electroporation was very efficient in inducing RNAi-mediated downregulation of ErbB3. Of particular interest, transfection of the T47D adherent breast carcinoma cell line with ErbB3 siRNA via electroporation caused a superior level of silencing of ErbB3 (data not shown) compared with the level of silencing achieved when using the transfection agent DMRIE-C. These data suggest that electroporation is an efficient method to induce RNAi in cells that appear to be resistant to siRNA transfection using commercial transfection agents. These data also suggest that electroporation may be an even more effective method of inducing RNAi in cells that are not resistant to transfection using commercial transfection agents. Wilda et al. (2002) were able to successfully transfect the nonadherent cell line K562 with siRNA using commercial carriers, suggesting that not all nonadherent cell lines will require transfection of siRNA via electroporation. However, our results raise the possibility that certain cell types may be uniquely sensitive to the mode of siRNA delivery and that electroporation may be a method to achieve more effective induction of siRNA, even in cell lines that are responsive to siRNA transfection using commercial carrier agents. The precise mechanisms underlying the success of the electroporation approach remain to be defined, but as alluded to, we speculate that this method is superior at facilitating access of the siRNA to the RNA-induced silencing complex.

Finally, we further assessed the specificity of RNAi-induced downregulation of ErbB3 by examining the expression levels of a variety of other surface molecules after treatment of the KAS-6/1 cells with the ErbB3 siRNA duplex. Figure 3A indicates that although ErbB3 expression was almost completely inhibited, surface levels of gp130, the IGF-1R, and VLA-4 were unaffected. To further test the specificity of RNAi in KAS-6/1 cells, we also treated these cells with dsRNA duplexes targeting survivin and the IGF-IR and compared the effects of these siRNA reagents with the ErbB3 siRNA on ErbB3 expression 24 hours after transfection. As can be seen in Figure 3B, downregulation of ErbB3 was observed only after transfection of ErbB3 siRNA, thus indicating the exquisite ability of this duplex to specifically silence ErbB3. Collectively, our results add to the literature demonstrating the feasibility of using RNAi in nonadherent cells and illustrate the importance of the method of cell transfection.

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